

AD \_\_\_\_\_

Award Number: DAMD17-99-1-9149

TITLE: The Role of Molybdenum Hydroxylase Generated Free Radicals  
in Alcohol Induced Breast Cancer

PRINCIPAL INVESTIGATOR: Richard Wright, Ph.D.

CONTRACTING ORGANIZATION: University of Colorado Health  
Sciences Center  
Denver, Colorado 80045-0508

REPORT DATE: September 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command  
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20010815 124

# REPORT DOCUMENTATION PAGE

OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE September 2000	3. REPORT TYPE AND DATES COVERED Annual (1 Sep 99 - 31 Aug 00)
----------------------------------	----------------------------------	---

4. TITLE AND SUBTITLE The Role of Molybdenum Hydroxylase Generated Free Radicals in Alcohol Induced Breast Cancer	5. FUNDING NUMBERS DAMD17-99-1-9149
6. AUTHOR(S) Richard Wright, Ph.D.	

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of Colorado Health Sciences Center Denver, Colorado 80045-0508  E-MAIL: richard.m.wright@uchsc.edu	8. PERFORMING ORGANIZATION REPORT NUMBER
---	---

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)  U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSORING / MONITORING AGENCY REPORT NUMBER
---	---

11. SUPPLEMENTARY NOTES
-------------------------

12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited	12b. DISTRIBUTION CODE
---	------------------------

13. ABSTRACT (Maximum 200 Words)  The breakdown of alcohol may contribute to the risk for breast cancer (BC) through the generation of toxic by-products of oxygen, (reactive oxygen species, "ROS") that can produce the DNA mutations and chromosome damage found in BC. ROS can be evolved stoichiometrically from cytochrome P450-E11 (CYP-E11) in the first step of alcohol degradation and subsequently by the action of xanthine oxidoreductase (XOR) and aldehyde oxidase (AOX) that consume acetaldehyde and NADH. We have proposed that these enzymes in the breast are responsible for the formation of ROS following alcohol consumption, and we suggest that ROS derived from alcohol will produce the DNA mutations and chromosome damage that lead to breast cancer. Our studies have shown that breast tissue expresses substantial levels of CYP-E11, XOR, and AOX. Furthermore, our recent studies have demonstrated that the genes encoding XOR and AOX are activated by cytokines and growth factors known to have significant effects on the mammary gland. Cytokine stimulated cultured epithelial cells produced ROS that could be blocked by inhibitors of XOR, hence activation of these enzymes generates intracellular ROS. We suggest that activation of ROS generating genes in the breast may contribute to alcohol mediated ROS toxicity leading to BC.
--

14. SUBJECT TERMS Breast Cancer	15. NUMBER OF PAGES 12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited
--	---	--	---

## Table of Contents

	Page
Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	4, 5
Key Research Accomplishments.....	6
Reportable Outcomes.....	6
Conclusions.....	6
References.....	7
Appendices.....	8-12

## Introduction

Alcohol consumption by women is one of several important risk factors for breast cancer (BC) that may promote BC through reactive oxygen species (ROS) induced carcinogenesis. Alcohol metabolism is known to produce ROS, and epithelial carcinoma of the breast is associated with high levels of hydroxyl radical (.OH) modified DNA, point mutations, single strand nicks, and chromosome rearrangement. Furthermore, ROS modification of DNA can produce the mutations and DNA damage found in many types of BC. ROS damage to breast DNA can be potentiated by accumulating iron and in conjunction with the diminished antioxidant defenses in breast tissue with advancing age thereby exacerbate the risk for ROS induced breast cancer.

Our preliminary studies had revealed that alcohol dehydrogenase (ADH), aldehyde oxidase (AOX) and xanthine oxidoreductase (XOR) are expressed and regulated in breast tissues. Mammary gland XOR and AOX were efficient sources of the ROS, hydrogen peroxide. Furthermore, XOR and AOX were found to generate ROS in two ways from alcohol metabolism: by *acetaldehyde* consumption and by an *intrinsic NADH oxidase* activity of XOR and AOX.

We proposed that: (1) expression of ADH and XOR or AOX in breast tissue provides the enzymes that generate ROS; (2) metabolism of alcohol produces acetaldehyde and NADH which can both be substrates for XOR or AOX and thereby result in ROS formation; and (3) ROS generated by XOR or AOX can induce the carcinogenic mutations and DNA damage found in breast cancer. The presence of elevated iron and diminished antioxidant status in breast tissue with greater age have provided additional support for the role of ROS in breast carcinogenesis.

## Body

*Our Original Technical Objectives And Current Status Are The Following:*

**To characterize ROS production by XOR and AOX purified from mouse mammary glands.**

Much of our last year was devoted to characterizing the ROS generating enzymes XOR and AOX from rat mammary glands. This enzymology component has focused largely on XOR which has been purified and characterized in the mammary gland. In addition, expression and regulation of XOR in mammary gland has been analyzed. Importantly, we have observed that XOR in the mammary gland is subject to regulation by lactogenic hormones. These two aspects of the present work, enzymologic characterization and regulation of XOR, were recently published (see below). Characterization of mammary gland AOX is in progress, and AOX has been purified from the gland tissue. Characterization of AOX gene expression and regulation has progressed significantly in the last year as described below. Our present plan will be to couple expression analysis and AOX dependent ROS generation in the next year. As described below expression analysis of AOX and XOR has suggested that each enzyme

may be uniquely regulated in the mammary gland and therefore responsible for ROS generation at different times.

**To determine the contribution of XOR and AOX to the damage and modification of DNA, specific proteins, and lipids in cultured mouse mammary epithelial cells and in whole mouse mammary glands.**

We began the studies directed at ROS mediated damage to mammary cells by first measuring ROS generation after activation of cells in culture. These experiments derived from those initiated in the last specific aim because they reflected the activation of XOR by additional mechanisms. We have measured the ROS sensitive fluorescence indicator dichlorofluorescein-diacetate (DCFH) in epithelial cells following activation by several cytokines thought to activate XOR or AOX. Presently, we find IL-1 inducible DCFH fluorescence can be inhibited in epithelial cells by allopurinol, an inhibitor of XOR. Thus XOR dependent ROS generation can be induced in epithelial cells by IL-1. This will have significant impact on the course of our experiments because the mammary gland and mammary epithelial cells are highly responsive to IL-1, and these data suggest that one consequence of this sensitivity is the generation of potentially cytotoxic ROS.

Alcohol metabolism is anticipated to modulate gene expression in epithelial cells and possibly expression of those genes important for alcohol breakdown and ROS generation. Accordingly, we have initiated analyses of the promoter regions for human XOR and AOX. Presently these studies indicate that XOR is subject to very complex regulation in epithelial cells. Transcription can be induced from promoter fusions by (a) lactogenic hormones, (b) IL-1, (c) IL-6, and (d) hypoxic growth. AOX is also subject to complex transcriptional activation. Its basal promoter is activated by Sp1/Sp3 transcription factors and the gene is sensitive to activation by several cytokines and growth factors. Importantly, in the mammary epithelium, AOX can be transcriptionally activated by TGF-beta and EGF, two growth factors with great significance to development in the mammary gland. Furthermore, regulation in the mammary gland is opposite to that occurring in lung epithelium. However, these studies do confirm induction of AOX and XOR gene expression in the mammary epithelium by cytokine and growth factors known to have important effects on the mammary gland and known to be affected by alcohol metabolism.

**To determine whether the metabolism of alcohol contributes to DNA and protein damage and modification resulting from the action of XOR and AOX.**

Studies aimed at examining DNA modification and mutagenesis during alcohol metabolism have not been started yet.

## **Key Research Accomplishments**

1. We have identified mouse and human mammary epithelial cells that express the ROS generators XOR and AOX in cell culture (HC-11 and HB-4a.).
2. We have cloned promoter domains for human XOR and human AOX and have begun to analyze mechanisms of transcriptional regulation in mammary epithelial cells.
3. We have detected ROS generation by XOR in epithelial cells using cytokine induced DCFH fluorescence.
4. We have purified XOR and AOX from mammary gland tissue in rats and mice and have extensively characterized XOR by enzymological criteria.

## **Reportable Outcomes**

1. Mammary gland and mammary epithelial cells in culture express genes responsible for ROS generation. Importantly, genes for both XOR and AOX were found to be activated in mammary glands and mammary epithelial cells.
2. The human XOR gene, encoding the ROS generating enzyme xanthine oxidoreductase, is activated in mammary epithelial cells by cytokines and hormones known to have significant effects in mammary gland development. Activation by cytokines was accompanied ROS generation that could be inhibited by XOR specific inhibitors. Thus, XOR has been found to be an inducible source of ROS in epithelial cells.
3. The human AOX gene, encoding an enzyme highly related to XOR and capable of significant ROS generation was also found to be expressed in mammary epithelial cells. Furthermore, its gene was found to be activated by a group of transcription factors, the Sp family, known to be important for mammary gland development and to be of particular significance for alcohol metabolism.

## **Conclusions**

The potential for AOX and XOR to contribute to ROS generation in the mammary gland and ultimately to BC has been strengthened in the last year. Both genes are activated in mammary glands and in mammary epithelial cells. Activation of each gene is distinct, therefore it is likely that they contribute to ROS generation at different times and following different stimulation. It is possible that either AOX or XOR may contribute to ROS generation in the mammary gland during alcohol metabolism and possibly under additional circumstances.

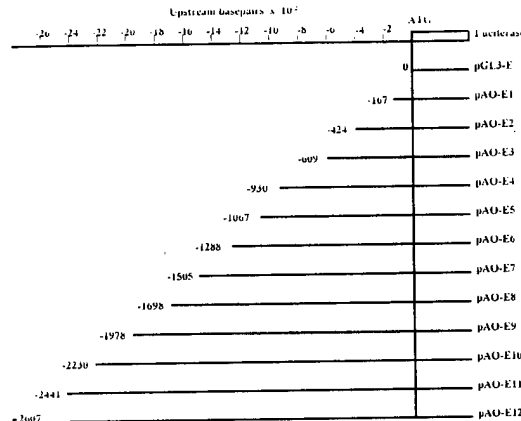
### References Published in the Last Year Under this Grant

29. **Wright, R.M.**, McManaman, J.L., and Repine, J.R.: Alcohol induced breast cancer - a proposed mechanism. **Free Radical Biology & Medicine** 26: 348-354, 1999.
30. **Wright, R.M.**, Clayton, D.A., Riley, M.G., McManaman, J.L., and Repine, J.E. cDNA cloning, sequencing, and characterization of male and female rat aldehyde oxidase (rAOX1). Differences in redox status may distinguish male and female forms of hepatic AOX. **J. Biol. Chem.** 274:2888-2998, 1999.
31. Faust-Chan, L-R., Hybertson, B., Flores, S., **Wright, R.M.**, and Repine J.R.: Initiation and tolerance to acute lung injury. A Tin-Yang mechanism involving interleukin-1. **Chest** 116:102S-103S, 1999.
32. McManaman, J.L., Neville, M.C., and **Wright, R.M.** Mouse mammary gland xanthine oxidoreductase: purification, characterization, and regulation. **Arch. Biochem. Biophys.** 371: 308-316, 1999.
33. McManaman, J.L., Hanson, L., Neville, M.E., and **Wright, R.M.**: Lactogenic hormones regulate xanthine oxidoreductase and beta casein levels in mammary epithelial cells by distinct mechanisms. **Arch. Biochem. Biophys.** 373: 318-327, 2000.
34. **Wright, R.M.**, Riley, M.G., Weigel, L.G., Ginger, L.A., Costantino, D.A., and MacManaman, J.L. Activation of the human aldehyde oxidase (hAOX1) promoter by tandem, cooperative Sp1/Sp3 binding sites. **DNA and Cell Biology** 19: 459-474, 2000.
35. **Wright, R.M.**, Ginger, L.A., McManaman, J.L., and Repine, J.E.: Characterization of a second locus for aldehyde oxidase (AOX) in humans: AOX gene duplication at chromosome 2q32.3-2q33.1 (**In preparation, 9/2000**).

## Appendices

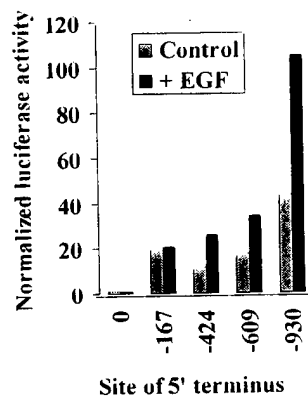
### A. The human AOX gene is activated in mammary epithelial cells by cytokine and growth factors.

#### 1. AOX promoter fusions to pGL3 luciferase reporter plasmid.

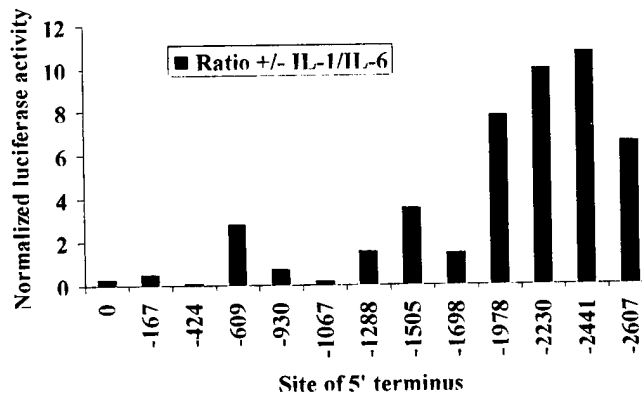


#### 2. AOX gene expression is activated by EGF in the -930 enhancer domain.

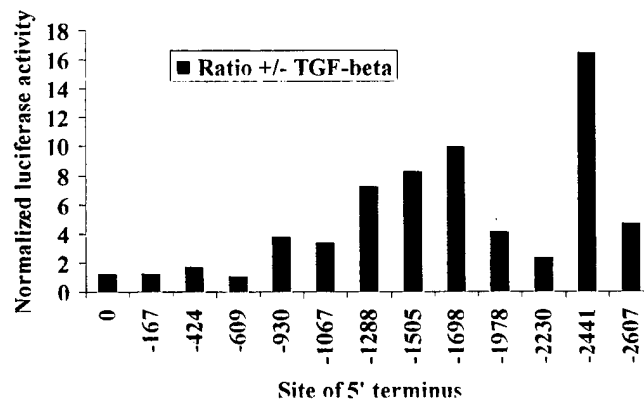
AOX1 Promoter Regulation by EGF in HC-11 Mammary Epithelial Cells



#### 3. AOX gene expression is activated by IL-1/IL-6 cocktail distal to the enhancer domain.



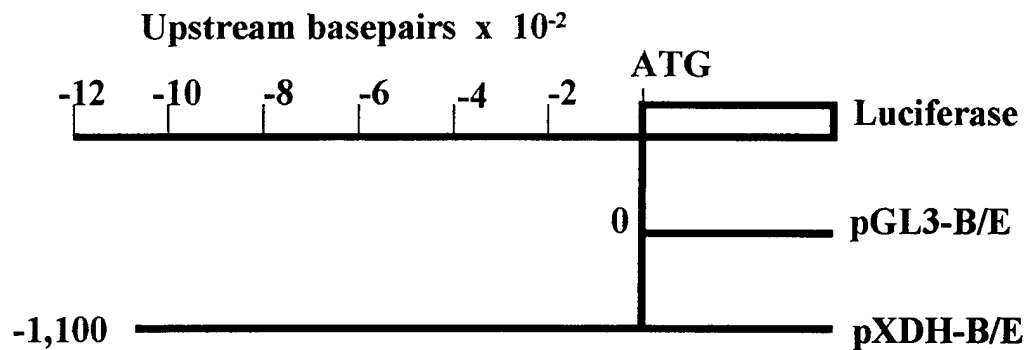
4. AOX gene expression is activated by TGF-beta in a region that includes the enhance and distal sites.



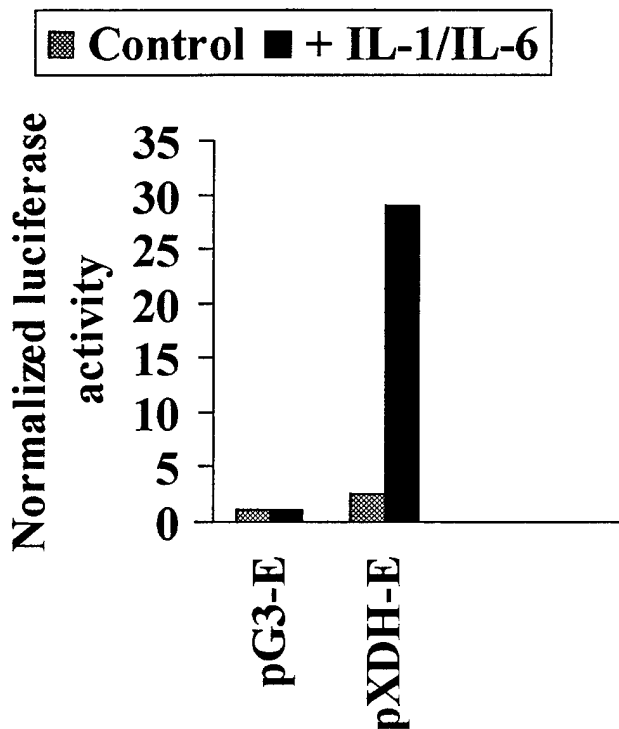
Fusions of the human AOX upstream DNA and promoter were constructed in the luciferase reporter gene pGL3 (Promega). Deletions were constructed (Panel 1) to enable localization of the proximal promoter and regions responsive to various inducers. EGF was found to activate expression from an enhancer at -930 nt (Panel 2); a cocktail of IL-1 and IL-6 activated in two distal regions at -1288 and -1978 (Panel 3); TGF-beta activated from within the -930 enhancer and showed progressive enhancement up to a silencer at -2230 nt (Panel 4). These data confirm activation of AOX in mammary epithelial cell by cytokines and growth factors and demonstrate that each activator functions by separate activation pathways.

**B. 1,100 base pairs of the XDH up-stream DNA were cloned and fused to the pGL3 luciferase reporter plasmid.**

1. The XDH fusion construct (n.b. deletions have not yet been constructed as shown above for AOX).

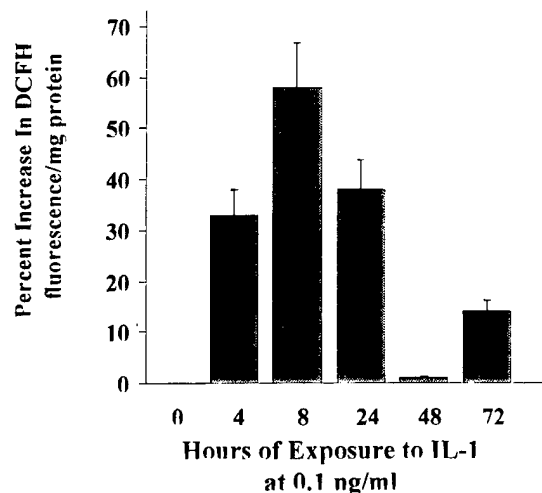


2. The XDH gene is dramatically activated by an IL-1/IL-6 cocktail.

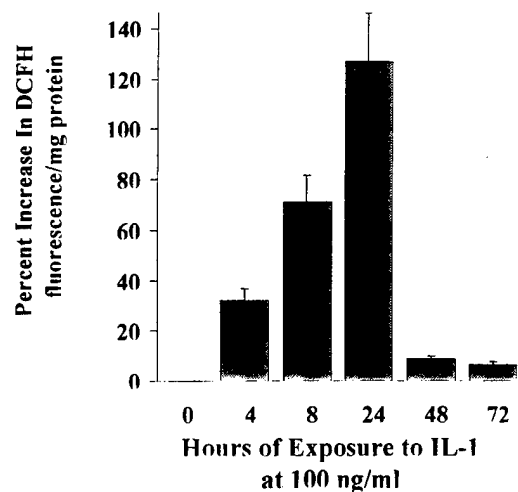


**C. IL-1 induces XDH dependent DCFH fluorescence in epithelial cells.**

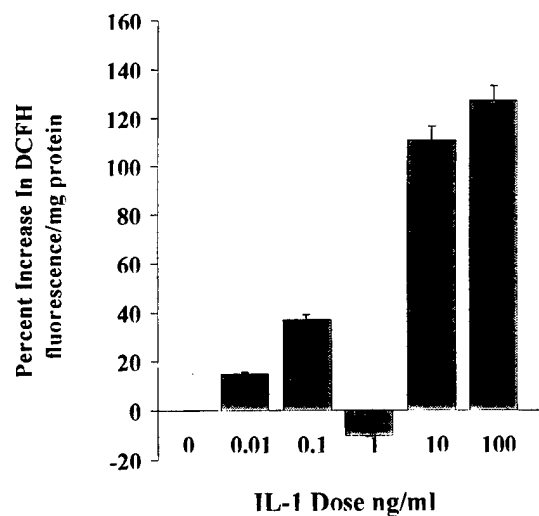
1. Time dependent increase in DCFH fluorescence following IL-1 stimulation at 0.1 ng/ml.



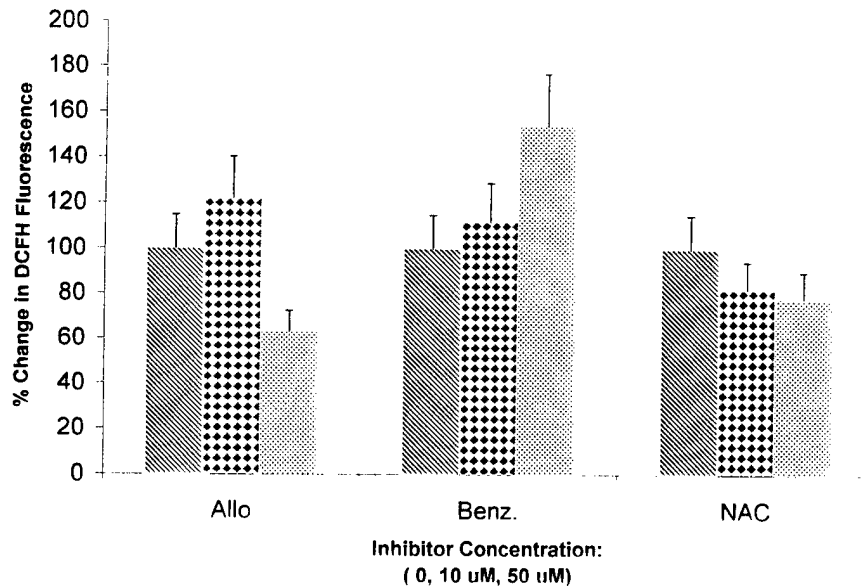
2. Time dependent increase in DCFH fluorescence following IL-1 stimulation at 100 ng/ml.



3. IL-1 dose dependent increase in DCFH fluorescence measured at 4 hours.



4. DCFH fluorescence is inhibited at 24 hours by an XDH inhibitor (Allopurinol), but not by an AOX inhibitor (Benzaldehyde). Low dose n-acetylcysteine showed inhibition of DCFH fluorescence. Higher doses of NAC are currently being tested.



Cultured epithelial cells were preloaded with the ROS indicator dichlorofluorescein-diacetate (DCFH) and were then treated with IL-1 for 0, 4, 8, 24, 48, or 72 hours using IL-1 doses of 0.1 ng/ml (Panel 1) or 100 ng/ml (Panel 2). The percent increase in DCFH fluorescence over the untreated cells was quantitated by fluorometric analysis and data were normalized to the untreated cell fluorescence. All data represent the average of 4 experiments and all error bars represent the standard deviation. IL-1 dose was titrated to 100 ng/ml (Panel 3), and it should be pointed out that the dose at 1 ng/ml is unlikely to represent a true biological response and may more likely represent an irregularity in the response or experimental error. ROS generation was partially inhibited by low dose n-acetylcysteine (NAC), and to a much greater extent by the XOR inhibitor, allopurinol (Allo). The AOX inhibitor, benzoquinone (Benz) did not inhibit IL-1 induced ROS generation. However, these data show that even in the absence of alcohol derived substrates, XOR can produce intracellular ROS that can be detected by the DCFH assay.